AGAR ELECTROPHORESIS

Part VI. Lipoproteins

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Abstract

Agar electrophoresis technique as described by Giri has been applied for the study of separation and estimation of serum lipoproteins in normal and pathological cases such as nephritis, diabetes, myxoedema and hypertensive heart diseases and in a few animals *viz.*, rat, rabbit, guinea pig, sheep, goat, cow and buffalo.

There are atleast two lipoprotein components revealed by staining with 0.1 per cent Sudan Black in 60 per cent ethanol in normal as well as in pathological human serum samples. The fast moving component has the mobility of serum albumin and the slow moving component has the mobility of α_1 globulin. They occupy about 30 per cent and 70 per cent of the total sudanophillic area respectively. In pathological cases there is a relative increase in the slow moving lipoprotein component.

In the case of animals, rabbit, goat and sheep sera showed three lipoprotein components and guinea pig serum only one. Buffalo serum has a greater percentage of fast moving component unlike the sera of other animals tested.

The suitability of the method for carrying out lipoprotein analysis in clinical laboratories is discussed.

Though the occurrence of lipoproteins in blood and other tissues has been well known, the interest in the study has developed only recently, particularly after the implication of these substances in the etiology of atherosclerosis and similar disorders of lipid metabolism [Gofman *et al.*, 1954]. In view of the complex nature of these substances, only complicated methods such as ultracentrifugation and salt or solvent precipitation at low temperature, were used for their study. With the advent of electrophoresis on stabilized media in the recent years, the fractionation and estimation of blood lipoproteins has been facilitated considerably in that even ordinary laboratories could undertake their investigation. Paper electrophoresis has been most extensively used by several workers, *viz.*, Fasoli [1952], Rosenberg [1952], Durrum *et al.* [1952], Swahn [1953], Nikkila [1953], Boyd [1954], Gottfried *et al.* [1954], Jenks *et al.* [1955] and Chapin [1956], whereas Kunkel and Slater [1952] and Ackermann *et al.* [1954] have reported the use of starch. Uriel and Graber [1956 and 1957] made use of agar as the stabilizing medium particularly with a view to develop immunoelectrophoresis.

The technique of agar electrophoresis as developed by Giri and his coworkers [1956 *a* and *b*; 1957 *a* and *b*] in this laboratory which has been successfully adapted for the study of serum proteins, animal hæmoglobins and mucoproteins, is used for the separation and estimation of lipoproteins in normal and pathological human sera and also in the sera of some animals. Only a preliminary report on this aspect was made previously by Giri [1957*c*] and now this present paper deals with the method and the advantages are discussed at the end.

Materials and Methods

The experimental procedure of agar electrophoresis used is the very same as described by Giri [1956*a*] except that the duration of experiment was 5 to 6 hours and the amount of serum spotted was 15 to 20 μl.

Staining solution:—The lipid soluble sudan dye [0.1g of Sudan III or IV or Sudan Black] is dissolved in absolute ethanol [60 c.c.] to which water [40 c.c.] is added. The mixture is heated just to boiling, cooled to room temperature and filtered under suction using Whatman No. 42 filter paper.

Staining procedure:—After electrophoresis, the agar plates are dried before a fan and immersed in the staining solution for 30 mts. The plates are then placed in the washing solution [50% ethanol] for 1-2 mts. With a couple of changes of the washing solution, the background colour on the plates disappears and lipoproteins appear as blue black [Sudan Black] or red [Sudan III or IV] bands on a colourless background. The plates are dried before a fan. Quantitative estimations are made by directly introducing these lipidograms into a photo-electric densitometer and the optical density measured as against the the stained area of the lipoproteins. The amount of lipoprotein is expressed as the percentage of the total sudanophillic area.

Total serum cholesterol is estimated by the method of Abell *et al.* [1952] and serum phospholipid, using Fiske and Subbarow reagent as suggested by Hawk *et al.* [1957]. Serum phospholipid is calculatsd as $P \times 25$ where P stands for phosphorus. Nitrogen is estimated by microkjeldahl method and protein is calculated as $N \times 6.25$.

Results

Typical agar electrophorograms stained for proteins with amidoschwarz 10 B and lipids with Sudan Black along with the curve showing the optical density as against the area occupied by each component are represented in Plate I.

In Table I and Plates II and III the lipid and lipoprotein distribution in sera of normal as well as a few tentatively diagnosed pathological cases such as nephritis, diabetes, myxoedema and hypertensive heart diseases is given.

Blood samples taken from a few animals have also been studied for lipid and lipoprotein distribution and the results obtained are illustrated in Table II and Plates IV and V.

TABLE I

Lipid and lipoprotein partition of human serum samples

No.	Age in Years	Sex	T. C.[a] mg %	P. L.[b] mg %	T. P.[c] g %	Percentage sudanophilic area	
						A	B
Normal cases							
1	I 26	M	176	253	6.95	32.5	67.5
2	K 27	M	187	238	6.90	32.0	68.0
3	R 24	M	190	218	7.69	32.0	68.0
4	B 24	M	172	211	6.86	38.0	62.0
5	SS 24	M	189	222	7.01	31.0	69.0
6	S 50	M	164	220	7.00	30.0	70.0
7	SR 23	M	186	237	6.87	27.0	73.0
8	G 23	M	190	238	6.85	33.0	67.0
9	RA 30	M	192	251	7.02	29.0	71.0
10	SA 18	M	174	217	7.05	31.0	69.0
11	D 25	M	191	247	7.40	21.0	79.0[d]
12	A 24	M	198	253	7.10	18.0	82.0[d]
13	H 26	M	207	257	6.98	18.0	82.0[d]
14	M 28	M	273	221	6.92	20.0	80.0[d]
	Average values with variation		182 ±8	230.5 ±22.5	7.02 ±0.67	31.55 ±6.45	68.45 ±6.45
Nephritis cases							
15	PA 23	M	180	235	4.53	36.0	64.0
16	HA 21	M	243	285	3.81	26.0	74.0
17	AS 15	M	488	305	4.08	13.0	87.0
18	EH 12	M	186	230	3.99	31.0	69.0
19	MU 8	F	265	243	4.16	21.0	71.0
Diabetes cases							
20	RI 10	F	209	299	6.3	12.0	88.0
21	SE 48	M	183	252	6.7	35.0	65.0
Myxoedema cases							
22	KU 30	F	232	239	7.9	20.0	80.0
23	BA 23	M	192	238	7.8	18.2	81.8
Hypertensive heart cases							
24	GE 45	M	312	253	7.4	28.5	71.5
BP. 180/110mm. Hg.							
25	MI 45	M	228	245	7.8*	27.4	72.6
BP. 170/140mm. Hg.							
26	MM 55	M	222	233	6.3	21.4	78.6
BP. 180/115mm. Hg.							
27	AR 61	M	284	250	6.5	20.0	80.0
BP. 208/100mm. Hg.							

[a] T.C.—Total serum cholesterol. [b] P.L.—Serum phospholipid. [c] T.P.—Total serum proteins.
[d] These serum samples were opaque as they were not taken during post-absorptive phase and hence are not considered while calculating the averages.

TABLE II

Lipid and lipoprotein partition in some animals

Species	T.P.[a] g%	T. C.[b] mg%	Number of lipoprotein components observed	% Sudanophillic area	
				A	B+C
Rat	6.5 ± 0.3	65 ± 15	2	15 ± 5	85 ± 5
Rabbit	6.3 ± 0.3	60 ± 10	3	28 ± 6	72 ± 6
G. Pig	6.1 ± 0.3	45 ± 15	1	—	—
Sheep	7.1 ± 0.2	115 ± 10	3	12 ± 5	88 ± 5
Goat	7.0 ± 0.3	105 ± 10	3	20 ± 5	80 ± 5
Buffalo	6.9 ± 0.3	100 ± 15	2	70 ± 10	30 ± 10
Cow	7.1 ± 0.3	110 ± 15	2	20 ± 10	30 ± 10
Human	7.4 ± 0.4	200 ± 30	2	35 ± 5	65 ± 5

[a] T. P.—Total serum protein.
[b] T. C.—Total serum cholesterol.

DISCUSSION

As represented in Plate I, at least two distinct lipoprotein components can be seen in human serum. The faster moving component [Lipoprotein A] occupies the position of albumin and the slow moving component [Lipoprotein B] occupies the position of α_1 globulin. There is no component occupying the position of β globulin in case of agar electrophoresis as is found in paper electrophoresis. The comparative increase in the mobility of lipoproteins on agar electrophoresis as compared with paper or starch electrophoresis may be on account of the low resistance offered by agar unlike other media. There is no trailing effect of the lipoprotein B in case of agar electrophoresis whereas in paper electrophoresis the trailing effect of β lipoprotein is inevitable on account of absorption of the dye by the paper. Lipoprotein B forms about 70% of the total sudanophillic area and the lipoprotein A forms the rest.

On perusal of Table I it can be noted that in normal cases the ratio of lipoprotein A to lipoprotein B is 30 : 70, whereas in pathological cases there is a relative increase in lipoprotein B. Even in normal sera [cases 11, 12, 13 and 14 in Table I] sometimes an increase in the lipoprotein B component is observed particularly when the sera is obtained from persons in the absorptive period, after a fatty diet. It is known that during the absorptive period there is a general increase of low density lipoprotein $S_f > 75$ [Gofman *et al.* 1954, *loc. cit.*] and as the absorption proceeds they are converted to the S_f 0-6 class or even the high density lipoproteins by the action of certain enzymes like lipoprotein lipases [Korn, 1955]. It is probable that lipoprotein A corresponds to the high density lipoprotein and the lipoprotein B to the low density lipoprotein.

Another point of interest is that there is a general relation between the

blood total cholesterol and the lipoprotein B levels. Though in most cases lipoprotein B increases with the increase in total cholesterol, this is not always true. It can be seen that certain samples showing low cholesterol levels have high lipoprotein B content [cases 20 and 23 in Table I] and *vice versa* [cases 16 and 24 in Table I]. While the number of cases reported here is very few to draw general conclusions, it nevertheless appears more desirable to measure the blood lipoprotein levels than merely the blood cholesterol levels in the diagnosis and prognosis of the disorders of liquid metabolism.

The study of the lipid and lipoprotein partition in the case of a few animals has shown the existence of 3 lipoprotein components in case of sheep, goat and rabbit serum, the mobility of the third component [lipoprotein C] being the same as that of normal α_2 globulin. Guinea pig has shown only one lipoprotein component. In case of most of the animals studied, the lipoprotein A component is present to a lesser extent than the liporprotein B component, but in case of buffalo it is *vice versa*. Bossak *et al*, [1954, *loc. cit.*] have shown in case of dog serum, a greater percentage of the fast moving lipoprotein fraction. The species differences observed are of interest. In case of rat serum a lipoprotein component with a mobility more than that of albumin is often encountered. Uriel and Grabar [1957, *loc. cit.*] working on a method of agar electrophoresis, particularly with a view to adopt it for immuno-electrophoresis, have reported the occurrence of a fast moving lipoprotein component called ρ lipoprotein to be present in very small quantities. It is also observed that the mobilities of lipoprotein on agar plate vary to some extent depending on the conditions of the experiment, particularly the voltage gradient and the current passing through the agar plate. It is therefore not possible to compare the results of other workers in view of the different conditions of experiment employed. At high currents [20-30 ma.] when a square plate [$7\frac{1}{2}'' \times 7\frac{1}{2}''$] is used, a faint band of lipoprotein similar to ρ lipoprotein has been observed, which is not seen in small plates [$2\frac{1}{2}'' \times 12''$, 200v, 4-5 ma./plate].

The advantages of agar electrophoresis for the study of lipoproteins are: [1] the short duration of electrophoresis staining and washing, the time taken for all these procedures being about 8-9 hours, unlike the procedures followed by Durrum *et al.* [1952, *loc. cit.*], Rosenberg [1952, *loc. cit.*] and Gottfried *et al.* [1954, *loc. cit.*], [2] the absence of trailing of lipoproteins unlike what is observed in paper electrophoresis [Swahn, 1952, *loc. cit.*] and [3] the presence of a total colourless background and transperancy of the pattern. Sudan Black seemed to be the most suitable dye and acetylation of sudan black and prestaining of lipoproteins as suggested by McDonald [1955] did not give satisfactory results. In case of starch electrophoresis, the plates cannot be directly stained and read in a densitometer.

From what has been discussed it is apparent that the agar electrophoresis technique can be usefully employed for the study of lipoproteins and in view of the simplicity and elegance of the method, it can probably be the method of choice in a clinical laboratory.

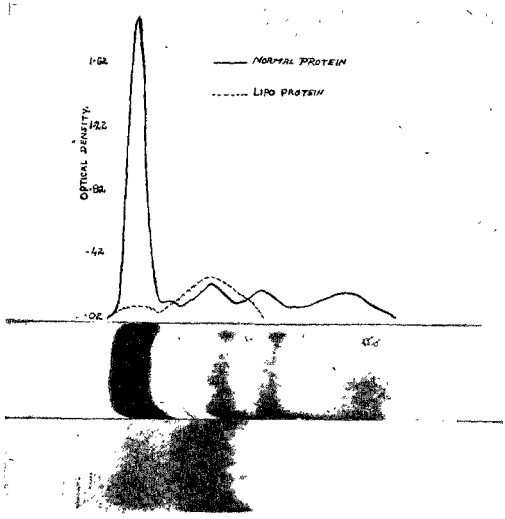

PLATE I

Agar electrophoretic Pattern of Human Serum

Agar electrophoretic patterns and corresponding densitometric curves of *(a)* Human serum normal proteins stained with amidoschwarz; *(b)* Human serum lipoproteins stained with sudan black

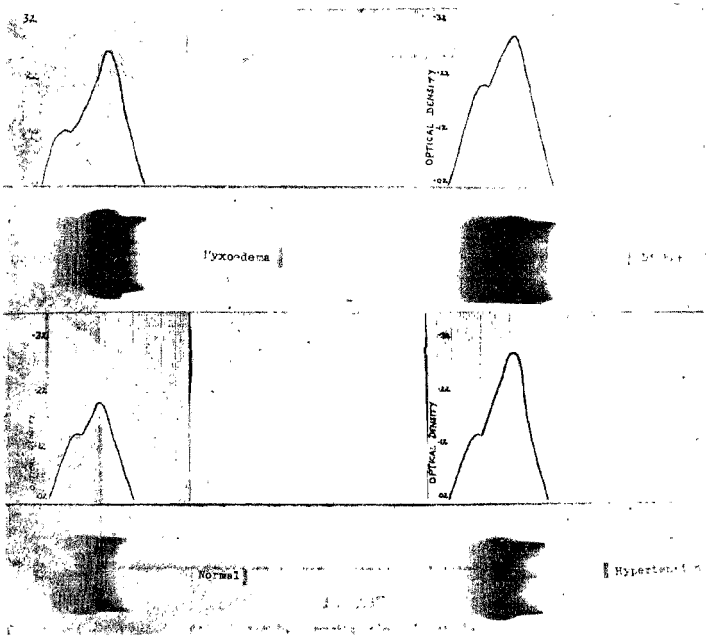

FIG. II

Agar Electrophoresis of Lipoproteins

Agar electrophorogram showing the distribution of lipoproteins in normal and pathological human sera

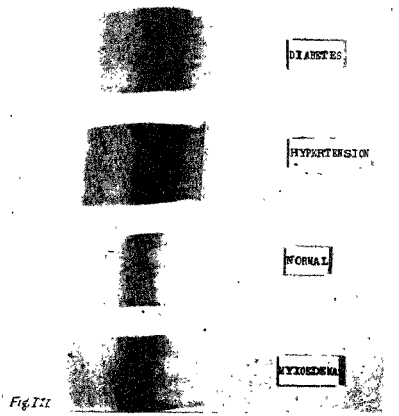

Fig. III

PLATE III

Comparative Agar Electrophoretic Patterns of Lipo-proteins from normal and Pathological Human Serum Samples

Conditions of experiment: Current 200 volts ; 36 milliamp; Duration of experiment : 5 hours ; Amount of sample spotted: 30 microlitres ; Buffer : Veronal-Sod. acetate ; pH 8.6.

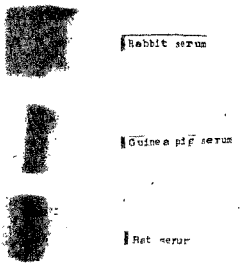

PLATE IV

Electrophoretic Patterns of Lipo-proteins on Agar Gel

Conditions of experiment : Current 200 volts, 36 mA : Buffer Veronal-Sodium accetate, pH 8.6, ionic strength 0 05 ; Duration of experiment—5 hrs. ; Aomunt of samples spotted 30 microlitres.

Electrophoretic Patterns of Lipo-proteins on Agar Gel.

PLATE V

Conditions of experiment :—Current 200 Volts, 36 milli-amps : Buffer Veronal-Sod-acetate, pH 8 6 ; Ionic strength 0 05 ; Duration of experiment 5 hours ; Amount of sample spotted 30 microlitres.

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